Genetic variation and division of *Pinus sylvestris* provenances by ISSR markers

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Abstract: Inter Simple Sequence Repeat (ISSR) was used to detect genetic variation among nine provenances, including 135 individuals. A total of 108 loci were amplified using 10 random primers. The differentiation of the percentage of polymorphic bands (PPB) among different provenances was evident, ranging from 27% to 54%, of which Honghuaerji provenance had the highest PPB and Kalunshan provenance had the lowest PPB. Shannon's Information index (I) at species level was 0.1581 and Nei's gene diversity (h) was 0.2393. Coefficient of gene differentiation (G_{ST}) calculated by Popgene was 0.3965, these results indicated that majority of genetic variation (60.35%) was found within provenances. According to dendrogram among *pinus sylvestriv* provenances, nine provenances were divided into two provenance areas, namely Daxing'an and Xiaoxing'an Mountains provenance area and Hulunbeier provenance area.

Keywords: ISSR; *Pinus sylvestris L.var mongolica litv*; Provenance; Genetic variation; Division of provenances
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Introduction

Pinus sylvestris, a variety of scots pine in China, is one of the major natural forest species in north of Daxing'an Mountains and Hulunbeier desert. It has the advantages of fast growth, good wood quality and strong adaptability, thus *Pinus sylvestris* has become the major afforesting tree species in Heilongjiang Province and west region in Northeast of China. The *Pinus sylvestris* has been extensively studied on biological characters (Vestgarden *et al.* 2001; 2003; Summers, 2002), genetic improvement (Gao 2001), management of seed orchard (Gomory *et al.* 2003), afforestation technique (Jia *et al.* 2003), wood quality (Persson *et al.* 1995), pest control (Meng *et al.* 2000; Xue *et al.* 1996), and provenance test (Liu *et al.* 1991), and so on.

ISSR (Inter Simple Sequence Repeat), with great repeatability and high polymorphism, have been widely used in the study of plant genetic variation. Yet up to now, few studies have been devoted to the genetic diversity of *Pinus sylvestris* by ISSR molecule markers.

In this study, the genetic diversity of provenances of *Pinus sylvestris* was analyzed with ISSR technique. The aims of this study are to detect the genetic variation between the provenances, and determine the genetic relationships among these provenances.

Materials and methods

Plant materials

Pinus sylverstris L. was sampled separately from nine provenances in Daxing'an Mountains, Xiaoxing'an Mountains, and Hulunbeier. Details on sampling sites were shown in Table 1. Leaves without diseases were collected randomly from the individuals with well growth potential, cleaned with distilled water, and then stored at –70℃ for molecular analysis.

Table 1. The sources of young leaves of *Pinus sylvestriv*

No.	Provenances	Code	Number of individuals
1	Gaofeng (Heilongjiang)	GF	15
2	Jinshan (Heilongjiang)	JS	15
3	Shibazhan (Heilongjiang)	SB	15
4	Mohe (Heilongjiang)	MH	15
5	Tuqiang (Heilongjiang)	TQ	15
6	Kalunshan (Heilongjiang)	KL	15
7	Honghuaerji (Inner mongolia)	HO	15
8	Handagai (Inner mongolia)	HD	15
9	Aershan (Inner mongolia)	AR	15

Extraction of total DNA

Total DNA of leaves was extracted by the method of Jiang (2003).

PCR reactions and detection of amplified products

The sequences of ISSR primers, provided by Columbia University of Canada, were synthesized by TaKaRa Company. A total of 60 primers were initially screened with the samples from three provenances. The screening criteria of primers for the same sample should have great repeatability, clear band and high polymorphism. Reaction system includes 1.5 mmol·L^{-1} $MgCl_2$, 0.2 mmol·L^{-1} dNTPs, 0.5 pmol·L^{-1} primer, 1×Taq DNA polymerase buffer, 1.5 units of Taq DNA (TaKaRa), and 40 ng template DNA in a total volume of 20 uL. PCR reactions were performed at 94℃ for 5 min, and then 94℃for 30 s, 56℃ for 45 s, 72℃ for 2 min for 35 cycles, and a final extension at 72℃ for 7 min. The PCR products were isolated by electrophoresis and photographed with UVP Gel Documentation Systems (GDS7600). Molecular weight was estimated using a 100 bp DNA ladder (MBI).

Data analysis

After electrophoresis, number 1 was used to indicate the presence of a single band in certain place, number 0 was used to indicate the absence of a single band, and the 0/1 matrixes were

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input into computer.

The phenotypic matrix can be obtained for further statistical analysis. To evaluate genetic variation with ISSR markers, the percentage of polymorphic bands (PPB) was calculated for each primer. Meanwhile, average number of alleles (A), number of effective alleles (Ne), Nei's genetic diversity (H), and Shannon's index of diversity (I) were separately calculated with the software of POPGENE32. Genetic structure of *Pinus sylverstris* L was analyzed with the software of STATISTICA.

Results

Genetic diversity analysis

Sixty ISSR primers were screened and 10 primers were selected according to the screening criteria of primer (Table 2).

Table 2. Sequences of 10 arbitrary primers

The list of primers	The sequences of primers	The list of primers	The sequences of primers
807	$(AG)_8T$	808	$(AG)_8C$
811	$(GA)_8C$	818	$(CA)_8G$
827	$(AC)_8G$	828	$(TG)_8A$
834	$(AG)_8YT$	835	$(AG)_8YA$
836	$(AG)_8YA$	848	$(CT)_8RC$

Note: R stands for A and T, Y stands for G and C, respectively.

A total of 108 bands (200-2200 bp) were amplified with these primers, with an average of 11 bands per primer (Fig. 1). The percentage of polymorphic bands (PPB) ranged from 27% to 54%, in which Honghuaerji provenance had the highest PPB and Kalunshan provenance had the lowest PPB. Thus, the highest and lowest genetic variation level existed in Honghuaerji and Kalunshan, respectively.

The number of effective alleles (Ne) is one of the indexes to evaluate genetic variation within provenances, and can reflect the polymorphism of provenances. The number of effective alleles of nine provenances ranged from 1.1339 to 1.3349, and the corresponding variation order of the provenances from the smallest to the biggest was Kalunshan < Mohe < Handagai < Shibazhan < Jinshan < Tuqiang < Aershan < Gaofeng < Honghuaerji. Genetic diversity of every population was evaluated with Shannon's index of diversity (I) and Nei's genetic diversity (H). The result showed that Shannon's index of diversity was in range of 0.0857-0.2006, and Nei's genetic diversity varied from 0.1340 to 0.3025. The results showed that the change trends of Shannon's index of diversity and Nei's genetic diversity for 9 provenances were both consistent with that of the number of effective alleles (Ne) (Table 3).

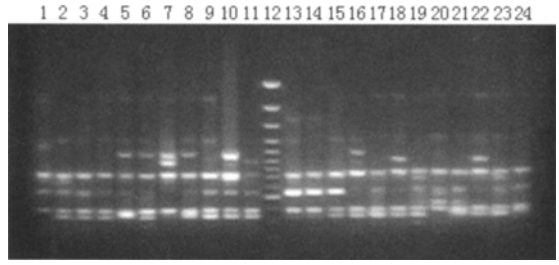

Fig.1 Profile of amplifying products by the primer 808

Note: Lane 1-11, 13-16 Honghuaerji provenance; Lane 17-24 Shibazhan provenance; Lane 12 Marker (MBI), the sizes from top to bottom are 3Kb, 2 Kb, 1.5 Kb, 1.2 Kb, 1 Kb, 0.9 Kb, 0.8 Kb, 0.7 Kb, 0.6 Kb, 0.5 Kb, 0.4 Kb, 0.3 Kb, 0.2 Kb and 0.1 Kb in order.

The genetic differentiation was estimated using Nei's index. Total genetic diversity (H_T) for polymorphic loci was 26.20% in tested provenances, in which diversity within provenances (H_S) was 15.81%, genetic diversity between provenances (D_{ST}) was 10.39%, and genetic differentiation (Gst) was 0.6035. The results indicated that there existed in a high level of genetic variation (60.35%) within provenances, and a low level of genetic variation (39.65%) between provenances.

Table 3. Comparison of genetic variation among nine provenances

Provenances	Total of ISSR loci	Number of polymorphic loci	Percentage of polymorphic bands (%)	Ne*	Shannon index (I)	Nei index (H)
Gaofeng	108	51	47%	1.3293	0.1955	0.2944
Jinshan	108	41	38%	1.2773	0.1587	0.2365
Shibazhan	108	41	38%	1.2506	0.1528	0.2334
Mohe	108	39	38%	1.2038	0.1244	0.1905
Tuqiang	108	53	49%	1.3182	0.1846	0.2762
Kalunshan	108	29	27%	1.1339	0.0857	0.1340
Honghuaerji	108	58	54%	1.3349	0.2006	0.3025
Handagai	108	40	37%	1.2129	0.1290	0.1967
Aershan	108	56	52%	1.3260	0.1920	0.2892
Mean		45	42%	1.2652	0.1581	0.2393

Note: *: Ne = Effective number of alleles (Kimura and Crow (1964))

Analysis of genetic clustering

To reveal the relationships between provenances, the Nei's genetic distance was calculated with the software of Popgene32 and a matrix of genetic distance was obtained. Subsequently, the matrix was analyzed with the software of STATISTICA and a dendrogram was generated.

At genetic distance of 0.16, nine provenances were classified into two groups (Fig. 2). The first group, namely Daxing'an Mountains and Xiaoxing'an Mountains provenance area, includes Kalunshan, Tuqiang, Mohe, Jingshan, and Shibazhan. The other group, namely Hulunbeier provenance area, includes Aer shan, Handagai, and Honghuaerji.

Discussion

Provenance experiment of *Pinus sylvestris* has been conducted in China since 1980s. On the basis of dominant ecological factors of natural distribution areas and phenotypic variation of parental population of *Pinus sylvestris*, the provenance distribution of *Pinus sylvestris* in northeast of China was studied in the

first time (Liu *et al.* 1991). Provenances of *Pinus sylvestris* were divided into two big areas and four small areas according to the results of peroxide isoenzyme, variance analysis, and major constituent analysis (Man *et al.* 1998).

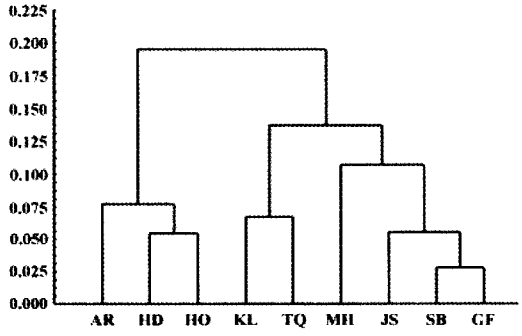

Fig. 2 Dendrogram among *pinus sylvestris L.* provenances based on the genetic distances

Note: AR is Aershan provenance; HD is Handagan provenance; HO is Honghuaerji provenance; KL is Kalunshan provenance; TQ is Tuqiang provenance; MH is Mohe provenance; JS is Jinshan provenance; SB is Shibazhan provenance; GF is Gaofeng provenance.

It is simple and easy to monitor genetic variability of *Pinus sylvestris* by morphology and phenotypic characteristic, however, the relationship between phenotype and genotype is complex due to gene expression and regulation, individual development and so on. And phenotype was usually affected by environment factors. Thus the change of phenotype didn't reflect the real genetic variability (Wang 2001). The number of loci monitored by the technology of isoenzyme was few, which can't represent the variability of the whole genome. Therefore, the divisions of provenance were imperfect by phenotype and isoenzymes.

According to the clustering of *Pinus sylvestris*, nine natural provenances of *Pinus sylvestris* were divided into two provenance areas at DNA level. One is Daxing'an Mountains and Xiaoxing'an Mountains provenance area, and the other is Hulunbeier provenance area.

On the basis of the estimation of gene differentiation coefficient (G_{ST}) and clustering analysis of genetic distance, we thought that the provenances of *Pinus sylvestris* in Daxing'an Mountains and Xiaoxing'an Mountains hadn't heterogeneous difference. It is might because both of them are mountain provenances and distribute in limited geographical areas. As a result, the selection pressures of habitat were consistent for them. The provenances of *Pinus sylvestris* in Hulunbeier are desert provenances. The habitat condition in Hulunbeier was different from those in Daxing'an Mountains and Xiaoxing'an Mountains. Therefore, the differences of selection pressures resulted in genetic differences in these two groups of provenances.

Many studies showed that the level of genetic differentiation between provenances of conifer was low (Li *et al.* 2003). The genetic variations mainly existed within provenances, just like *Pinus sylvestris*. The percentage of the variation within provenance was 60.35, possibly because pollen of *Pinus sylvestris* has air chamber and may pollinate freely, which result in many hybrids in the population and obvious segregation in its offsprings.

The analysis of clustering picture for genetic distance of *Pinus sylvestris* showed that genetic distance was closest between Gaofeng and Shibazhan provenances, which was consistent with the results of isoenzyme clustering by Jie *et al.* (1995). *Pinus sylvestris* in Gaofeng was artificial forest, which was planted for a long time, and local people don't know whether the accurate geographical source of seeds was Jinshan or Shibazhan. According to the clustering resulted from analysis at DNA level, we deduced artificial forest of *Pinus sylvestris* might come from Shibazhan provenances.

References

Gao Fengshan, Wei Jianhua, Hu Yingge,*et al.* 2001. Summary on genetic improvement of *Pinus sylvestris* var. *mongolica* [J]. Journal of Liaoning Forestry Science & Technology, **3**: 5–8. (in Chinese)

Gomory, D., Bruchanik, R., Longauer, R. 2003. Fertility variation and flowering asynchrony in *Pinus sylvestris*: consequences for the genetic structure of progeny in seed orchards [J]. Forest Ecology and Management, **174**: 117–126.

Hamelin, R.C., Beaulieu, J., Plourde, A. 1995. Gemetoc diversity in population of *Cronartium ribicola* in plantations and natural stands of *Pinus strobes* [J]. Theo Appl Genet, **91**(8): 1214–1221.

Jia Tiemin, Meng Linghua. 2003. Afforestation technique of *Pinus sylvestris* var. *mongolica* in Desert of Hulunbeir [J]. Journal of Hulunbeir College, **11**(1): 90–123. (in Chinese)

Jiang Jing. 2003. Experiment Theory and Technique of Molecular Biology [M]. Harbin: Northeast Forestry University Publishing House, p28.

Jie Qiming, Zhang Minli, Wang Jiang, *et al.* 1995. Genetic Construction of Natural Population for *Pinus sylvestris* var. *mongolica* [J]. Forestry Science & Technology, **20**(1): 1–6. (in Chinese)

Li Bin, Gu Wanchun. 2003. Review on Genetic Diversity in *Pinus* [J]. Hereditas, **25**(6): 740–748.

Liu Guifeng, Yang Shuwen, Lv Qingyou, *et al.* 1991. Study on The Provenance Test of *Pinus sylvestris* var. *mongolica* (I) -Division of Provenance areas [J]. Journal of Northeast Forestry University, **19**: 103–107. (in Chinese)

Liu Guifeng, Yang Shuwen, Xia Dean, *et al.* 1991. Study on The Provenance Test of *Pinus sylvestris* var. *mongolica* (III) -Regular of Geographical Variation [J]. Journal of Northeast Forestry University, **19**: 95–102 (in Chinese)

Liu Guifeng, Yang Shuwen, Zhang Jing, *et al.* 1991. Study on The Provenance Test of *Pinus sylvestris* var. *mongolica* (II) –Biosysystematics [J]. Journal of Northeast Forestry University, **19**: 92–94. (in Chinese)

Man Xiuling, Cao Jifu, Yao Yujun, *et al.* 1998. The application of peroxidase isoenzy on species source divisions of *Pinus sylvestris* var. *mongolica* [J]. Bulletin of Botanical Research, **18**(1): 83–89. (in Chinese)

Meng Xiangzhi, Ji Yuhe, Shen Xiufeng. 2000. Study on Biological Characteristics of *Pissodes* sp. [J]. Jinlin Forestry Science and Technology, **29**(6): 6–12. (in Chinese)

Persson, B., Anders Persson, Erik G. Stahl, Ulf Karlmats. 1995. Wood quality of *Pinus sylvestris* progenies at various spacing [J]. Forest Ecology and Management, **76**: 127–138.

Summers, R.W. 2002. Cone size of Scots pines (*Pinus sylvestris*) in the Highlands of Scotland-inplications for pine-eating crossbills [J]. Forest Ecology and Management, 164: 303–305

Vestgarden, L.S. 2001. Carbon and nitrogen turnover in the early stage of Scots pine (*Pinus sylvestris L.*) needle litter decomposition: effects of internal and external nitrogen [J]. Soil Biology & Biochemistry, **33**: 465–474.

Vestgarden, L.S., Selle, L.T., Stuanes, A.O. 2003. In situ soil nitrogen mineralisation in a Scots pine (*Pinus sylvestris L.*) stand: effects of increased nitrogen input [J]. Forest Ecology and Management, **176**: 206–216.

Wang Mingxiu. 2001. Forestry Genetic and Breeding [M]. Beijing: China Forestry Publishing House, p94. (in Chinese)

Xue Yu , Cheng Dongsheng, Han Xiaoyun, *et al.* 1996. Aeciospore morphology and EST isozyme analysis of scotch pine needle rust disease Pathogeny-Coleosporium [J]. Journal of Northeast Forestry University, **24**(6): 47–49. (in Chinese)